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Protein Stationary Phases

J. Haginaka, Mukogawa Women's University, Nishinomiya, Japan

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In the early 1950s, it was reported that the binding of the enantiomers of an anionic azo-dye to bovine serum albumin (BSA) or human serum albumin (HSA) was different. Further, it was reported that L-tryptophan was bound to serum albumin (bovine mercaptoalbumin) more strongly than the D-form. These studies clearly indicated the possibility of enantioselective binding of ligands to proteins. In 1973, BSA-Sepharose was used for the separation of tryptophan enantiomers; this is the first report on the use of a protein stationary phase for chiral resolution purposes. With this stationary phase, D- and L-tryptophan were clearly resolved, and the D-form was eluted first, as shown in the previous binding studies in solution. In the following years, high performance liquid chromatography (HPLC) chiral stationary phases based on a protein were developed and used to separate a variety of enantiomers. HPLC chiral stationary phases based on a protein are of special interest because of their unique properties of stereoselectivity and because they are suited for separating a wide range of enantiomeric mixtures. Protein-based stationary phases developed so far have included albumins such as BSA and HSA, enzymes such as trypsin, α-chymotrypsin, lysozyme and pepsin, and glycoproteins such as α_1-acid glycoprotein (AGP) from human or bovine serum, cellobiohydrolase I (CBH I), ovomucoid (in fact, ovoglycoprotein), avidin, ovotransferrin and flavoprotein (riboflavin-binding protein). The physical properties of these proteins are shown in **Table 1**. Chiral stationary phases based on BSA, HSA, pepsin, AGP, CBH I, ovomucoid and avidin are now commercially available. Among those, AGP and ovomucoid-based stationary phases can separate a wide range of weakly acidic, weakly basic and neutral racemates.

The advantages of protein-based stationary phases generally include the use of an aqueous mobile phase, enantioselectivity for a wide range of compounds and direct analysis without derivatization. The disadvantages included low capacity, lack of ruggedness and limited understanding of the chiral recognition mechanism. Thus, the protein-based stationary phases are useful for analytical purposes, but are not generally applicable to preparative isolation. To stabilize the protein-based stationary phases, chemical modification of the side chains of the amino acids in the protein has been tried. Further, chiral stationary phases based on a protein fragment or protein domain have been prepared. These can be of higher capacity because only the active protein mass is used. Also, it is possible to understand the chiral recognition sites of protein-based stationary phases by investigating whether or not independent chiral binding sites exist on each fragment or domain.

This article deals with the preparation of HPLC chiral stationary phases based on a protein, their chiral recognition properties and the chiral recognition mechanisms of these stationary phases.

Preparation of HPLC Chiral Stationary Phases Based on a Protein

Generally, a protein is bound to derivatized silica gels. The disadvantage of silica-based stationary

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Table 1 Physical properties of proteins

Protein	*Molecular mass (kDa)*	*Carbohydrate composition (%)*	*Isoelectric point*	*Origin*
Albumins				
BSA	66		4.7	Bovine serum
HSA	66		4.7	Human serum
Enzymes				
Trypsin	24		10.1	Bovine pancreas
α-Chymotrypsin	25		8.1–8.6	Bovine pancreas
Pepsin	34		< 1	Porcine stomach
Lysozyme	14		10.5–11.0	Egg white
Glycoproteins				
α_1-Acid glycoprotein	44	45	2.7	Human or bovine serum
Cellobiohydrolase I	60	6	3.6	Fungus
Ovoglycoprotein	30	25	4.1	Egg white
Avidin	68	7	10.0	Egg white
Ovotransferrin	77	2.6	6.1	Egg white
Flavoprotein	32–36	14	4	Egg white or yolk

phases is that the eluent pH is limited to the ranges 2–8. However, in a strong acidic or alkaline solution, a protein sometimes suffers from denaturation. The separation of enantiomers on a protein-based stationary phase is generally attained using an eluent whose pH is between 3 and 8. Thus, the limitation of eluent pH ranges originated from silica-based materials is no problem for the use of protein-based stationary phases. **Figure 1** shows the typical preparation method for protein-based HPLC chiral stationary phases; in part A the method includes activation of porous aminopropylsilica gels by *N*,*N*′-disuccinimidylcarbonate (DSC), binding of a protein and blocking of the activated amino groups. In this case, a side chain amino group(s) of a protein such as lysine and arginine and/or an *N*-terminal amino group could be used for binding the protein to the activated gel. On the other hand, using water-soluble carbodiimide and *N*-hydroxysulfosuccinimide (HSSI), the carboxyl group of a protein can be bound to aminopropylsilica gels, as shown in **Figure 1**B.

Further, proteins can be bound to aminopropylsilica gels using glutaraldehyde as a cross-linker, resulting in cross-linking by Schiff-base formation. The resulted imino functions are reduced by using sodium cyanoborohydride. Glycerylpropylsilica gels activated with 1,1′-carbonyldiimidazole have been used for the preparation of chiral stationary phases. According to the two methods described above, an amino group of a protein is used to bind to the derivatized silica gels. In addition, a protein can be physically adsorbed on to porous silica gels. The disadvantage of the adsorption method is that the adsorbed protein can be eluted, and it is better to bind a protein covalently to the base materials in order to avoid losses. The chiral recognition properties of a bound or adsorbed protein may be different from those of the protein in solution because of blocking of functional groups and/or conformational changes. The bound protein is often more stable to the changes of eluent pH and eluent composition compared to the protein in solution.

Retention and Enantioselectivity of Solutes on Protein-based Stationary Phases, and Optimization of Resolution

Table 2 shows the influence of eluent pH on the retention and enantioselectivity of various solutes on AGP-based chiral stationary phases. The retention factor of a basic solute, metoprolol, increased with an increase in the eluent pH. The decrease in the retention factor of an acidic solute, 2-phenoxypropionic acid, is ascribable to ion exclusion in addition to ionic repulsion between the carboxyl group of the 2-phenoxypropionic acid and the negatively charged AGP with an increase in eluent pH. The retention of basic solutes should be due to electrostatic interactions with the positively charged solutes and the negatively charged protein, in addition to hydrophobic interactions. Although the retention factor of an uncharged solute (ethotoin, hexobarbital) shows almost no pH dependence, a slight increase is observed with increasing eluent pH. This increase might be

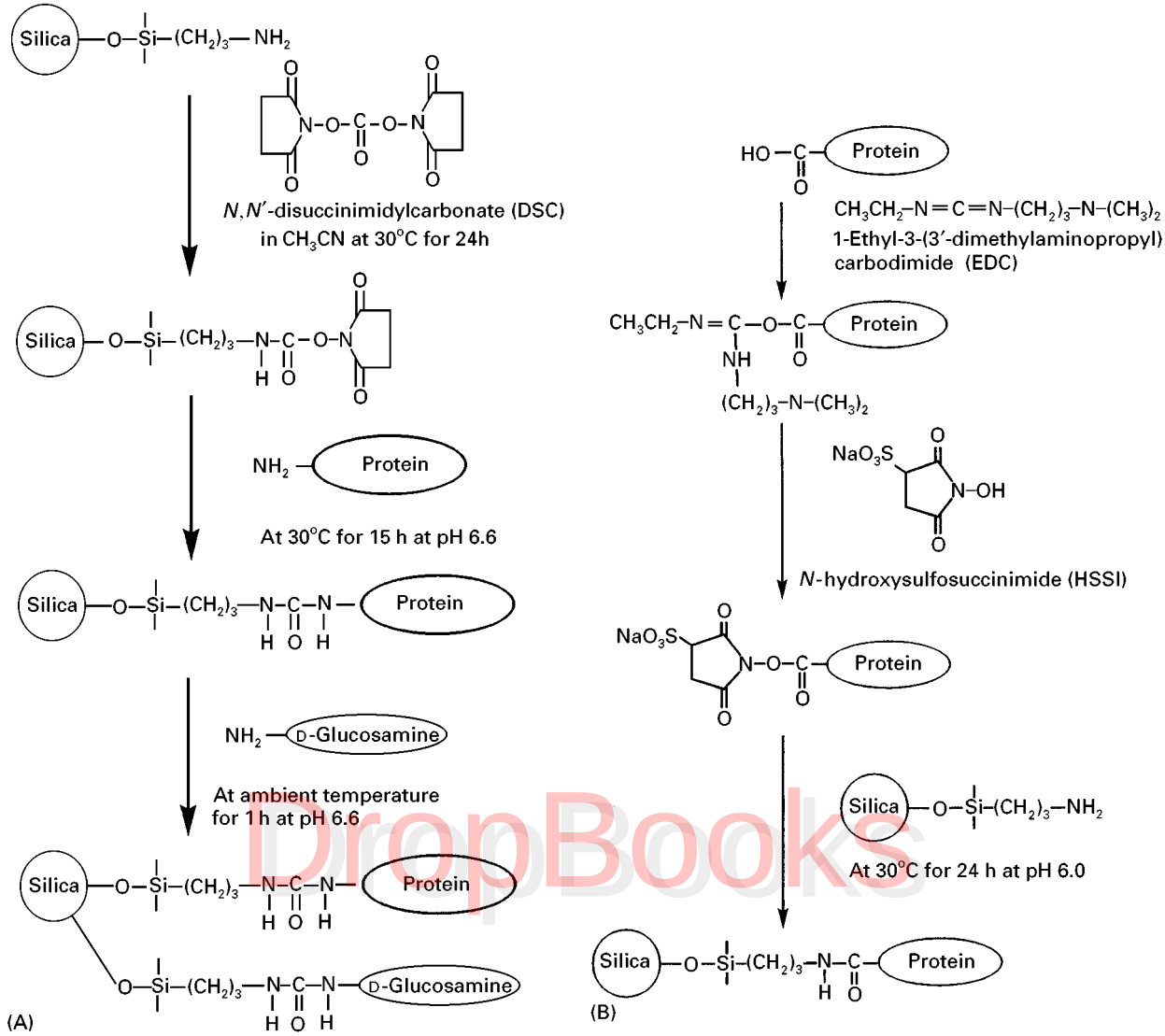

Figure 1 Synthesis scheme for the preparation of protein-based stationary phases: (A) via an amino group of a protein; (B) via a carboxyl group of a protein.

due to changes in the binding properties of the protein resulting from conformational changes. **Table 3** shows the influence of the 2-propanol content on retention and enantioselectivity of various solutes on AGP-based chiral stationary phases. With an increase in the 2-propanol content, the retention and

Table 2 Influence of eluent pH on retention of enantioselectivity of various solutes on AGP-based chiral stationary phase

Solute	*pH 4.5*		*pH 5.5*		*pH 6.5*		*pH 7.5*	
	k_1	α	k_1	α	k_1	α	k_1	α
2-Phenoxypropionic acid	8.55	1.59	1.77	1.57	0.32	1.48		
Ethotoin	4.06	3.82	3.87	4.19	3.82	4.59	4.13	5.06
Metoprolol	0.40	1.25	2.20	1.29	9.23	1.42	22.5	1.48
Hexobarbital	9.39	1.44	9.47	1.47	10.3	1.66	11.6	2.10

Mobile phase, 0.01 mol L^{-1} phosphate buffer; k_1 is the retention factor of the first eluted enantiomer; α is enantio separation factor $= k_2/k_1$, where k_2 is the retention factor of the second eluted enantiomer. (Reproduced with permission from Hermansson J (1989) Enantiomeric separation of drugs and related compounds based on their interaction with α_1-acid glycoprotein. *Trends in Analytical Chemistry* 8: 251.)

Table 3 Influence of 2-propanol on the retentivity and enantioselectivity of various solutes on AGP-based chiral stationary phase

Solute	2-*PrOH* (%)									
	1		2		4		6		8	
	k_1	α	k_1	α	k_1	α	k_1	α	k_1	α
Disopyramide					8.51	3.70	3.62	3.37	1.77	3.20
Chlorpheniramine	11.2	2.34	7.35	1.71	4.59	1.38				
Mepensolate			6.35	1.54	2.65	1.40	1.42	1.38	1.00	1.21
Mepivacaine	26.0	1.36	10.7	1.31	4.42	1.33	2.48	1.36	1.58	1.35
Bupivacaine					18.6	1.70	8.84	1.72	5.01	1.74

Mobile phase, 2-propanol in phosphate buffer, pH 7.2; k_1, retention factor of the first eluted enamtiomer; α, enantioseparation factor $= k_2/k_1$, where k_2 is the retention factor of the second eluted enantiomer. (Reproduced with permission from Hermansson J (1989) Enantiomeric separation of drugs and related compounds based on their interaction with α_1-acid glycoprotein. *Trends in Analytical Chemistry* 8: 251.)

enantioselectivity of solutes are decreased. These results suggest that hydrophobic and electrostatic interactions play an important role in the retention and enantioselectivity of racemic solutes on AGP-based columns. Further, the hydrogen bonding properties of the organic modifier influence enantioselectivity to a large extent. As shown in **Figure 2**, verapamil enantiomers are not resolved on the AGP-based column using 1-propanol as an organic modifier, but are resolved using acetonitrile.

Similar retentive and enantioselective properties are observed with other protein-based stationary phases. As described above, hydrophobic, electrostatic and hydrogen bonding interactions play an important role in chiral recognition of solutes on these phases. Thus, enantioseparations of solutes can be optimized by changing eluent pH, and the type and content of the uncharged organic modifier. Sometimes, charged modifiers such as *N*,*N*-dimethyloctylamine and octanoic acid are used for the enantioseparation of a charged solute. **Figure 3** shows a scheme for the optimization procedure for ovomucoid-based stationary phases.

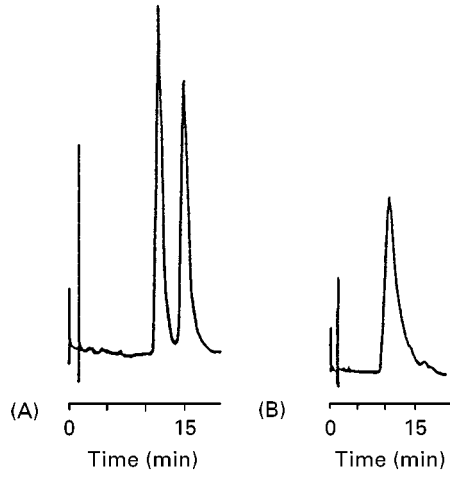

Figure 2 Influence of the nature of the organic modifier on the enantioselectivity of verapamil on an AGP-based column. HPLC conditions: column, Chiral-AGP (4.0 mm i.d. × 100 mm); eluent: (A) 10% acetonitrile in 0.01 mol L^{-1} phosphate buffer, pH 7.0; (B) 4% 1-propanol in 0.01 mol L^{-1} phosphate buffer, pH 7.0. (Reproduced with permission from Hermansson J (1989) Enantiomeric separation of drugs and related compounds based on their interaction with α_1-acid glycoprotein. *Trends in Analytical Chemistry* 8: 251.

Albumin-based Stationary Phases

BSA and HSA are closely related proteins and, consequently, the chromatographic properties of the chiral stationary phases based on these proteins are similar. Sometimes the elution order is reversed between chiral stationary phases based on these proteins; on the HSA-based phases (*S*)-warfarin elutes before (*R*)-warfarin, whereas on the BSA-based phases the opposite elution order is observed.

A variety of weakly acidic and neutral compounds are resolved on chiral stationary phases based on BSA and HSA. 2-Arylpropionic acid derivatives such as naproxen, flurbiprofen, ibuprofen, ketoprofen and fenoprofen, reduced folates such as leucovorin and 5-methyltetrahydrofolate, and benzodiazepines such as oxazepam, lorazepam and temazepam are separated. **Figure 4** shows enantioseparations of leucovorin, lorazepam hemisuccinate and *N*-benzoylphenylalanine on an HSA-based column. However, cationic compounds are not resolved on BSA and HSA phases. The structure-binding relationship for benzodiazepines using HSA stationary phases reveals that the binding of benzodiazepines occurs at a site that contains both a hydrophobic pocket and an area

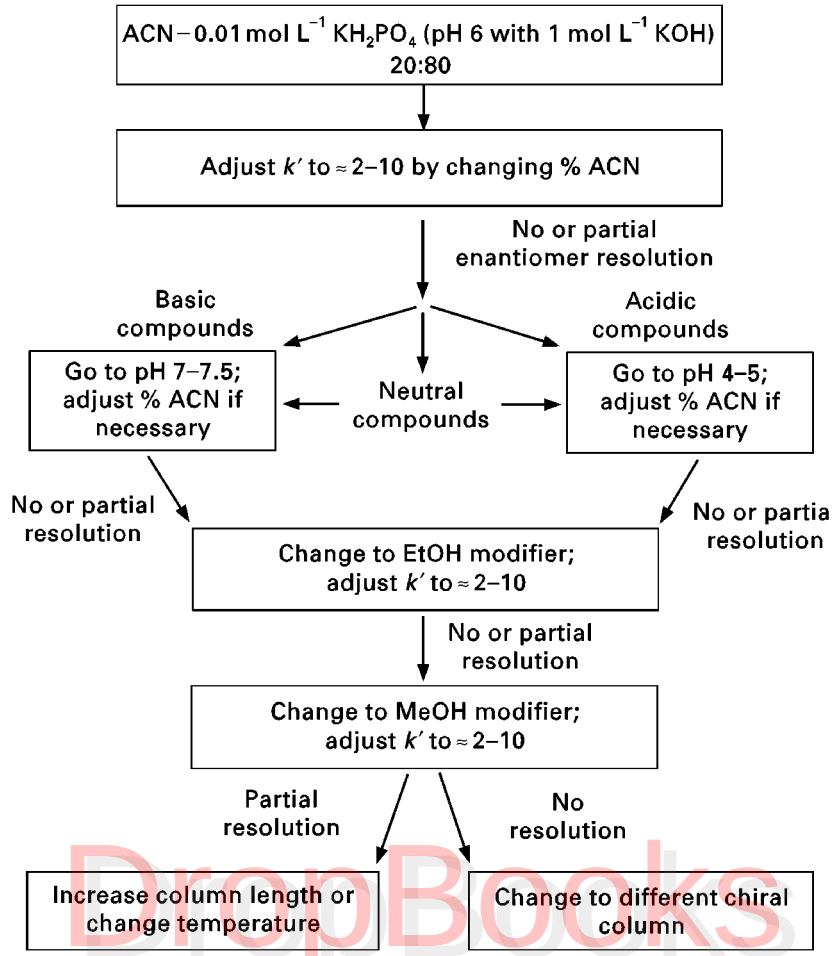
ACN – 0.01 mol L^{-1} KH_2PO_4 (pH 6 with 1 mol L^{-1} KOH)
20:80
Adjust k' to ≈ 2–10 by changing % ACN
No or partial enantiomer resolution
Basic compounds
Acidic compounds
Go to pH 7–7.5; adjust % ACN if necessary
Neutral compounds
Go to pH 4–5; adjust % ACN if necessary
No or partial resolution
No or partial resolution
Change to EtOH modifier; adjust k' to ≈ 2–10
No or partial resolution
Change to MeOH modifier; adjust k' to ≈ 2–10
Partial resolution
No resolution
Increase column length or change temperature
Change to different chiral column

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Figure 3 Scheme for the optimization procedure for ovomucoid-based stationary phases. ACN, acetonitrile. (Reproduced with permission from Kirkland KM and McCombs DA (1994) Changes in chiral selectivity with temperature with an ovomucoid protein-based column. *Journal of Chromatography A* 666: 211.)

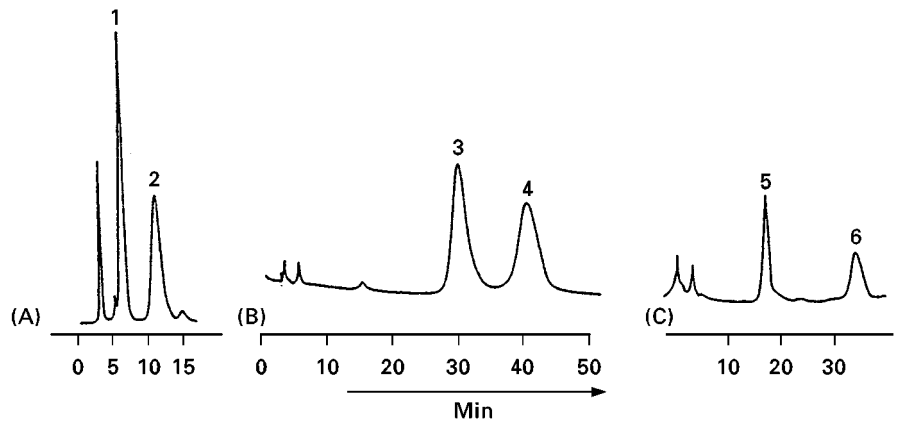

Figure 4 Enantioseparations of (A) leucovorin, (B) lorazepam hemisuccinate and (C) *N*-benzoyl-phenylalanine on an HSA-based column. HPLC conditions: column, 4.6 mm i.d. × 150 mm; eluent, 50 mmol L^{-1} phosphate buffer (pH 7.0): 1-propanol (94 : 6, v/v); flow rate, 0.8 mL min^{-1}. Peaks: 1,(6*S*)-leucovorin; 2, (6*R*)-leucovorin; 3, (−)-(*R*)-lorazepam hemisuccinate; 4, (+)-(*S*)-lorazepam hemisuccinate; 5, *N*-benzoyl-D-phenylalanine; 6, *N*-benzoyl-L-phenylalanine. (Reproduced with permission from Domenici E, Bertucci C, Salvadori P *et al.* (1990) Synthesis and chromatographic properties of an HPLC chiral stationary phase based upon human serum albumin. *Chromatographia* 29: 170.)

of cationic charge, and that the chiral recognition occurs in this binding site.

Enantioselectivity of stationary phases based on BSA produced with isolated protein fragments has been investigated. The BSA fragment following peptic digest of BSA has molecular weights of about 35 kDa which is an *N*-terminal half of amino acid residues 1–307. The BSA fragment phases give longer retentions for benzoin and benzodiazepines, and higher enantioselectivity for lorazepam, benzoin and fenoprofen because of a higher density of chiral recognition site(s), compared with native BSA phases. **Figure 5** shows chromatograms of lorazepam enantiomers on BSA and BSA fragment-based columns. However, it is plausible that the conformation of the BSA fragment might be different from that of the native BSA.

Enzyme-based Stationary Phase

Trypsin and α-chymotrypsin are a family of serine proteases. Trypsin-based stationary phases can resolve *O*-, *N*,*O*-derivatized amino acids which are substrates of the enzyme. This means that chiral separations are due to the activity of the enzyme, and that the chiral recognition site is on the enzyme activity site. α-Chymotrypsin stationary phases can resolve amino acids and amino acid derivatives.

When the eluent of pH 7 is continuously delivered, the pepsin-based stationary phases lose their chiral recognition properties. This result reveals that the immobilized pepsin is irreversibly denatured above pH 7. Thus, the use of an eluent with pH less than 6 is recommended. By using a mixture of phosphate buffer and organic modifier as an eluent, cationic (especially β-blocking agents) and neutral enantiomers are resolved, while no resolution of acidic enantiomers is observed. **Figure 6** shows enantioseparations of

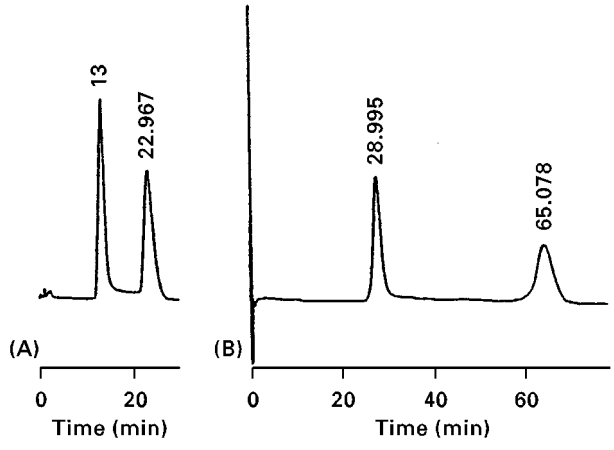

Figure 5 Chromatograms of lorazepam enantiomers on (A) BSA-based and (B) BSA-fragment-based columns. HPLC conditions: column, 2.1 mm i.d. × 100 mm; eluent, 50 mmol L^{-1} phosphate buffer (pH 7.5) containing 4% 1-propanol; flow rate, 0.2 mL min^{-1}. (Reproduced with permission from Haginaka J and Kanasugi K (1995) Enantioselectivity of bovine serum albumin-bonded columns produced with isolated protein fragments. *Journal of Chromatography A* 694: 71.)

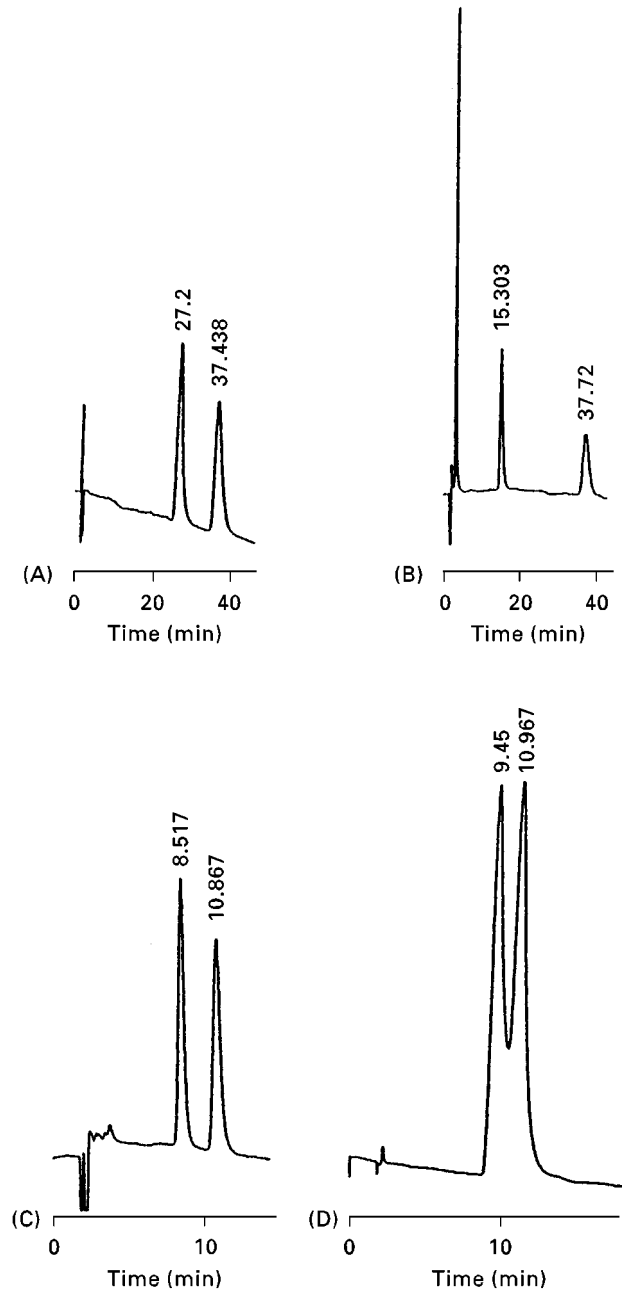

Figure 6 Enantioseparations of (A) homochlorcyclizine, (B) verapamil, (C) alprenolol and (D) oxazepam on a pepsin-based column. HPLC conditions: column, 4.6 mm i.d. × 100 mm; eluent, 20 mmol L^{-1} phosphate buffer (pH 5.1) containing (A) 5 and (B) 10% acetonitrile and (C) and (D) 5% ethanol; flow rate, 0.8 mL min^{-1}. (Reproduced with permission from Haginaka J, Miyano Y, Saizen Y *et al.* (1995) Separation of enantiomers on a pepsin-bonded column. *Journal of Chromatography A* 708: 161.)

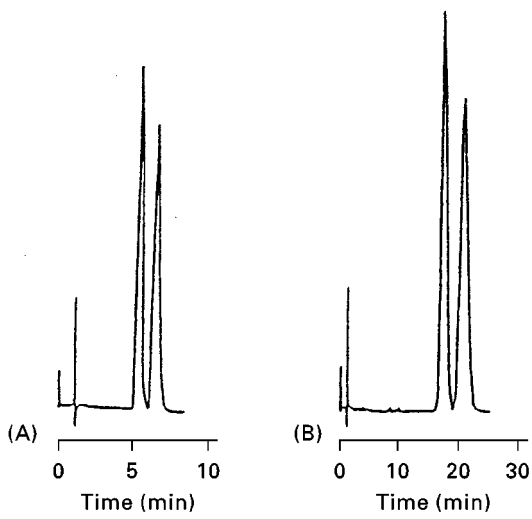

Figure 7 Enantioseparations of (A) metoprolol and (B) pindolol on an AGP-based column. HPLC conditions: column, Chiral-AGP (4.0 mm i.d. × 100 mm); eluent (A) 3.8% ethanol in 0.01 mol L^{-1} phosphate buffer, pH 7.0; (B) 15% methanol in 0.01 mol L^{-1} phosphate buffer, pH 7.0. (Reproduced with permission from Hermansson J (1989) Enantiomeric separation of drugs and related compounds based on their interaction with α_1-acid glycoprotein. *Trends in Analytical Chemistry* 8: 251.)

homochlorcyclizine, verapamil, alprenolol and oxazepam on a pepsin-based column. Also, lysozyme-based stationary phases showed chiral recognition ability to cationic and neutral solutes but no resolution of anionic solutes.

α_1-Acid Glycoprotein-based Stationary Phase

AGP is the major plasma protein responsible for the protein binding of basic drugs. A variety of cationic, anionic and uncharged compounds can be resolved using this stationary phase. Cationic compounds resolved include *β*-blockers such as alprenolol, oxprenolol, propranolol, metoprolol and pindolol and histamine antagonists such as chlorpheniramine and dimethindene. **Figure 7** shows enantioseparations of metoprolol and pindolol on an AGP-based column. Anionic compounds resolved include 2-arylpropionic acid derivatives such as fenoprofen, ibuprofen and naproxen. As described above, separation of enantiomers is optimized by changing eluent pH, and the type and content of the uncharged organic modifier. However, cationic modifiers such as *N*,*N*-dimethyloctylamine and tetrabutylammonium bromide and anionic modifiers such as octanoic acid and butylic acid can be used effectively with AGP-based materials. An ion-pairing modifier, *N*,*N*-dimethyloctylamine, in the eluent gives a large increase in enantioselectivity of racemic acid, naproxen, on an AGP-based stationary phase, where the retention factor of the second-eluted enantiomer is increased drastically. This is due to an allosteric interaction in which the affinity of the protein for the enantiomer is increased by the addition of the modifier.

AGP consists of a protein domain and sugar moieties, both of which have chiral components. It was thought that drug binding to AGP occurred at a single hydrophobic pocket or cleft within the protein domain of the molecule. However, the role of sugar moieties on enantioselective binding by AGP has not been investigated. It has been reported that sialic acid residues influence the enantioselective binding of basic drugs in different ways. They are not involved in the enantioselective verapamil–AGP binding. On the other hand, they participate in the binding of (*S*)-propranolol but not of (*R*)-propranolol. Further studies are required to clarify the role of sugar moieties in chiral recognition and the chiral recognition mechanism of AGP.

Cellobiohydrolase-based Stationary Phases

Chiral stationary phases based on CBH I can resolve acidic and basic racemates into their enantiomers. Higher enantioselectivity is especially obtained for the separation of *β*-blocking agents such as propranolol, oxprenolol and metoprolol. **Figure 8** shows a chromatogram of propranolol enantiomers on a CBH I-based column.

CBH I has a structural organization with a terminal, 36 residue-long binding domain connected to the rest of the enzyme (i.e. the core) through a flexible arm. The interconnecting region is rich in serine, threonine and proline residues and is highly glycosylated. The core is enzymatically active. CBH I is enzymatically degraded into two fragments, core

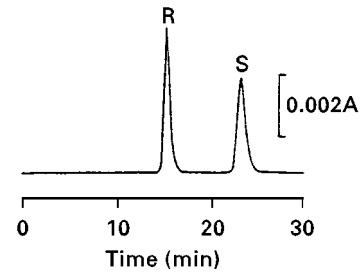

Figure 8 Chromatogram of propranolol enantiomers on a CBH I-based column. HPLC conditions: column, 5.0 mm i.d. × 250 mm; eluent, sodium-acetate buffer pH 4.7, I = 0.01, containing 0.5% 2-propanol; flow rate, 0.3 mL min^{-1}; detection, 254 nm. (Reproduced with permission from Erlandsson P, Marle I, Hansson L *et al.* (1990) Immobilized cellulase (CBH I) as a chiral stationary phase for direct resolution of enantiomers. *Journal of the American Chemical Society* 112: 4573.)

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and binding domain. Each fragment has been shown to contain at least one enantioselective site for propranolol. The dominating enantioselective site for propranolol and other solutes is located on the core, the main part of the enzyme. The three-dimensional structure of the active site of CBH I has been elucidated by X-ray crystallography, and it has been shown that the binding site is a tunnel with the dimensions $0.4 \times 0.7 \times 4$ nm. There are seven acidic amino acid residues, four tryptophan residues and also, tyrosine, serine, threonine, arginine and histidine lining the tunnel. This gives the prerequisite for obtaining stereoselective binding of a broad range of chiral solutes.

Ovoglycoprotein-based Stationary Phases

Chiral stationary phases based on chicken ovomucoid (OMCHI) from egg whites have been prepared, which show chiral recognition abilities for a wide range of cationic, anionic and uncharged compounds, similar to AGP phases. However, the chiral recognition ability of OMCHI comes from other glycoproteins, as described below.

Ovomucoid from turkey egg whites (OMTKY) and OMCHI, which exist as three tandem, independent domains, have been isolated, purified and characterized, and columns based on OMTKY and OMCHI domains have been made to test their chiral recognition properties. The third domain of OMTKY and OMCHI consists of glycosylated (OMTKY3*S* and OMCHI3*S*) and unglycosylated domains (OMTKY3 and OMCHI3). The OMTKY3 and OMTKY3*S*, and OMCHI3 and OMCHI3*S* are enantioselective to at least two classes of compounds, benzodiazepines and 2-arylpropionic acid derivatives. Glycosylation of the third domain does not affect chiral recognition. The chiral recognition mechanism of the OMTKY3 has been elucidated using nuclear magnetic resonance measurements, molecular modelling and computational chemistry. The selected binding model for each of the (*R*)- and (*S*)-enantiomers of U-80 413 (whose structure is illustrated in **Figure 9**), a 2-arylpropionic acid derivative, with OMTKY3 shows similarities and differences in orientation and intermolecular interactions between the (*R*)- and (*S*)-enantiomers. The carboxyl groups of each enantiomer engage in electrostatic interactions with the positive charge on arginine-21. The carbonyl group on U-80 413's central ring shares a hydrogen bond with the NH_3^+ group of lysine-34. The distinguishing difference between the enantiomers is the proximity of the phenyl group of the (*R*)-enantiomer and phenylalanine-53. However, neither the first nor the second domain of the OMTKY and a

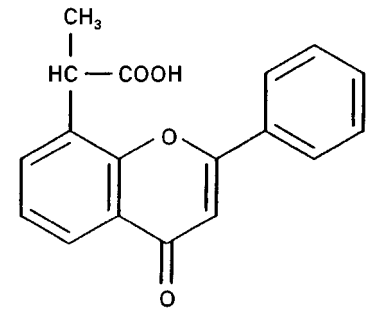

Figure 9 Structure of U-80 413.

combination of the first and second domains, or the second domain of the OMCHI gives chiral recognition ability.

These results suggest that three domains may be needed to work in concert for chiral recognition of various solutes, because columns made with the whole, intact OMTKY and OMCHI can resolve a wide range of weakly acidic, weakly basic and neutral racemates. Recently, a new protein from chicken egg whites has been isolated and characterized. It is termed ovoglycoprotein (OGCHI, which means ovoglycoprotein from chicken egg whites). It is found that 10% of OGCHI is included in crude OMCHI preparations. OMCHI and OGCHI columns made from isolated pure proteins have been compared with regard to their chiral recognition abilities. It is found that the pure OMCHI gives no chiral recognition abilities, and that the pure OGCHI gives better chiral recognition than those of the impure OMCHI reported previously, as shown in **Table 4**.

Table 4 Comparison of retention factor (k_1), enantioselectivity (α) and resolution (Rs) of various solutes on columns made with crude OMCHI and isolated OGCHI

Compound	*Column*					
	Crude OMCHI			*OGCHI*		
	k_1	α	*Rs*	k_1	α	*Rs*
Benzoin	2.50	2.71	6.06	11.4	3.18	10.1
Hexobarbital	0.35	1.00		1.52	1.29	0.83
Alprenolol	2.53	1.12	0.31	15.9	1.13	0.84
Propranolol	7.49	1.12	0.44	42.6	1.18	0.78
Chlorpheniramine	1.03	2.05	3.00	5.42	2.27	5.89
Ibuprofen	4.05	1.18	0.88	9.03	1.39	2.58
Ketoprofen	7.69	1.11	0.82	23.5	1.20	1.97

All values were averages of three replicates. HPLC conditions: column, 2.0 mm i.d. × 100 mm; eluent, 20 mmol L^{-1} phosphate buffer (pH 5.1)-ethanol 90 : 10 (v/v); column temperature, 25°C; flow rate, 0.2 mL min^{-1}; detection, 220 nm. (Reproduced with permission from Haginaka J, Seyama C and Kanasugi N (1995) The absence of chiral recognition ability in ovomucoid: ovoglycoprotein-bonded HPLC stationary phases for chiral recognition. *Analytical Chemistry* 67: 2539.)

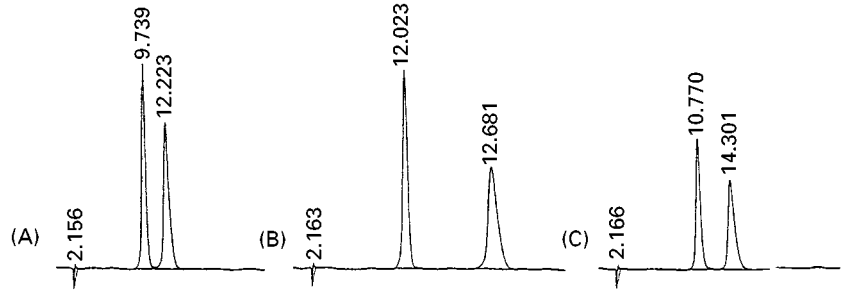

Figure 10 Enantioseparations of (A) ibuprofen, (B) ketoprofen and (C) flurbiprofen on an avidin-based column. HPLC conditions: column, 4.6 mm i.d. × 150 mm; eluent, 20 mmol L^{-1} potassium phosphate (pH 6.5) containing 6% ethanol; flow rate, 1.2 mL min^{-1}. (Reproduced with permission from Miwa T, Miyakawa T and Miyake Y (1988) Characteristics of an avidin-conjugated column in direct liquid chromatographic resolution of racemic compounds. *Journal of Chromatography* 457: 227.)

Though only 10% OGCHI was included in the impure OMCHI, the impure OMCHI-based column gave moderate chiral recognition (**Table 4**). This is due to the fact that OGCHI is preferentially bound to DSC-activated aminopropyl-silica gels compared with OMCHI, despite similarity in their average molecular masses (30 and 27 kDa, respectively).

Other Protein-based Stationary Phases

A number of further minor protein-based stationary phases for HPLC, based on avidin, ovotransferrin and flavoprotein, have been described. Avidin, a basic protein, strongly binds biotin with an association constant of 10^{15} mol L^{-1}. Avidin-based stationary phase shows excellent chiral recognition ability for 2-arylpropionic acid derivatives such as ibuprofen, ketoprofen, flurbiprofen, pranoprofen and fenoprofen. **Figure 10** shows enantioseparations of ibuprofen, ketoprofen and flurbiprofen on an avidin-based column. A biotin-bound avidin stationary phase does not exhibit chiral recognition ability, because the biotin modifies the structure of the avidin.

Ovotransferrin is labile to heat and acid, but it seems that it is stable to heat when combined with metal ions such as iron, copper, manganese and zinc. Ovotransferrin is further stabilized by conjugation to silica gel as a chiral stationary phase, and ovo-transferrin-based stationary phases have been used for the separation of a basic compound, azelastine.

Flavoprotein, the riboflavin-binding protein, in egg whites and yolks has been introduced as a chiral stationary phase for HPLC. Egg white and yolk flavoproteins appear to be the product of the same gene but to have undergone different post-translational modifications. The amino acid sequence is the same but the yolk flavoprotein is between 11 and 13 amino acids shorter. There are differences in the carbohydrate links between flavoproteins from egg yolks and whites. Chiral stationary phases based on flavoproteins from egg whites and yolks exhibit chiral recognition abilities for uncharged, anionic and cationic compounds. There is no evidence whether the drugs interact with the riboflavin-binding site or not.

Future Trends

Chiral recognition sites of protein-based stationary phases will be located and their chiral recognition mechanisms will be elucidated using X-ray crystallography, ^{1}H-nuclear magnetic resonance spectroscopy and computational chemistry. Based on these findings, a protein, protein fragment or protein domain having chiral recognition abilities or a point-mutated protein can be overexpressed by genetic technologies. In the future, we could make protein-based stationary phases, which have better chiral recognition abilities and higher loadability, and are more stable than those so far prepared.

See also: **III/Chiral Separations:** Cellulose and Cellulose Derived Phases; Cyclodextrins and Other Inclusion Complexation Approaches; Ion-Pair Chromatography; Liquid Chromatography; Liquid Exchange Chromatography; Molecular Imprints as Stationary Phases.

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Supercritical Fluid Chromatography

N. Bargmann-Leyder and M. Caude, Laboratoire de Chimie Analytique (unité de recherche associée au CNRS) de l'Ecole Supérieure de Physique et Chimie Industrielles de Paris, Paris, France
A. Tambuté, Centre d'Etudes du Bouchet, Le Bouchet, France

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Introduction

Great emphasis is currently placed on differences in biological activities, potencies and toxicities of enantiomeric pharmaceutical compounds. The US Food and Drug Administration (FDA) has recently implemented regulations for the enantiomeric purity of enantiomeric drugs and chemicals. This has led to the development of chromatographic methods for the enantiomeric resolution of racemates including gas chromatography, liquid chromatography, and more recently supercritical fluid chromatography (SFC).

The physicochemical properties of enantiomers are the same except when they are placed in an asymmetric environment. This can be obtained before the chromatographic column or within the column by using a chiral derivatizing agent in the mobile phase or by using a chiral stationary phase.

Formation of Diastereomers by Using a Pre-Column Derivatization

In this method, the racemate is reacted with an optically pure compound leading to formation of diastereomers. Owing to their different physiocochemical properties, diastereomers can be resolved by using classical achiral mobile and stationary phases. This method can only be applied to molecules bearing reactive functions such as amines, acids and alcohols. For preparative purposes, partial racemization can occur when recovering the initial enantiomer. This problem represents the major limitation of this method. Moreover, this method has some disadvantages: (1) the chiral reagent must be optically pure, or its optical purity has to be well known (otherwise, poor accuracy will be achieved); (2) the derivatizing reaction must be quick and quantitative; and (3) the chromatographic behaviour of the derived diastereomers should be suitable (easy separation, good stability under the chromatographic conditions, ease of recovery with absence of racemization during the step leading to the initial enantiomers).

This was the method of choice before the development of chiral stationary phases (CSPs). It is still applied, but usually in order to improve detection limits. The method is not commonly used with SFC although (*S*)-trolox methyl ether has been used to derivatize chiral alcohols for attempted separation by GC and SFC with achiral systems. Using this derivatizing method, several compounds were successfully resolved by SFC but GC failed because of thermal decomposition of the ester derivatives.

Formation of Labile Diastereomers in the Mobile Phase

This method generally consumes chiral reagent. Moreover, the major limitation concerns detection, which must be compatible with the nature of the chiral reagent contained in the mobile phase. In the case of preparative applications the limitation is related to the recovery of the sample, which must be separated from the chiral reagent. Although, the optical purity of the reagent has no effect on the accuracy of the results, it decreases the selectivity of the method. One example of the use of SFC in this way is the chiral separation of amino alcohols using chiral ion pairing (**Figure 1**). In this case SFC analysis time was significantly less than that for high performance liquid chromatography (HPLC) separation.